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RESEARCH PAPERS

Detection of a new variant of *Citrus tristeza virus* in Greek citrus crops

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Summary. *Citrus tristeza virus* (CTV), the most destructive virus of citrus, is a quarantine pathogen in Greece. Since 2000, several accidental imports of infected propagation material have been detected in the country, and while eradication measures were applied, a few disease foci still remain. CTV isolates were collected from Chania (Crete) and the "lemonwood" of Poros (Peloponnese), and their genetic variability was studied using single-strand conformation polymorphism (SSCP). One previously characterized isolate from Argolida grafted on a Mexican lime (GR3) and two Italian isolates from Calamondin were also included in the study. ELISA and RT-PCR tests confirmed CTV presence, and SSCP analysis of the virus amplified coat protein (CP) gene was used to separate either distinct virus isolates for cloning the CP gene or variants (haplotypes) for sequencing. Analyses showed that selected variants of four representative isolates clustered into three of the seven defined phylogenetic groups: groups 3b and 5 (severe isolates) and group M (mild isolates). The prevalent haplotypes detected in the CTV from lemonwood of Poros (GR9) were in group 3b, confirming previous results. However, one sequence variant was identified as a recombinant between haplotypes from groups 3b and 5. Variants of these two groups were also detected in the Italian Calamondin isolate. In the grafted Mexican lime isolate (GR3) from Argolida, only one haplotype was found which belonged to group M, while in the field isolate from Chania (GR6) the only haplotype detected was in group 5. This is the first report of variants of group 5 in Greece, suggesting an unknown virus introduction. The prevalence of severe isolates in the area is of particular concern, and implications for the future of the CTV epidemics are discussed.

Key words: CP gene, CTV isolates, nucleotide diversity, phylogenetic analysis, SSCP, recombination.

Introduction

Citrus tristeza virus (CTV) (genus *Closterovirus*, family *Closteroviridae)* is the most economically important and damaging virus of citrus trees (Moreno and Garnsey, 2010). This virus is listed among the largest positive stranded RNA viruses of higher plants; its 19.3 kb positive sense ssRNA genome is encapsidated mainly by the p25 capsid protein (CP) and the p27 proteins, in thread-like filamentous particles of size about 2000 × 11 nm (Moreno *et al.*, 2008).

CTV worldwide dispersal occurred via the movement of infected plant material, often grafted on sour orange rootstocks (*Citrus aurantium* L.) used to control *Phytophthora* root rots. Local spread of the virus is mainly by aphids in a semi-persistent manner (Moreno *et al.*, 2008). Virus spatial and temporal spread depends on the aphid vector species present (Gottwald *et al.*, 1999). In Europe, virus epidemics are associated with the presence of *Aphis gossypii* Glover (Cambra *et al.*, 2000), while the less competent vectors *A. spiraecola* (Patch) and *Toxoptera aurantii* (Boyer de Fonscolombe) also occur (Hermoso de Mendoza *et al.*, 1988; Cambra *et al.*, 2000). *T. citricida* (Kirkaldy), the most efficient vector of CTV (Yokomi *et al.*, 1994) is established in Asia, Australia, sub-Saharan Africa, Central and South America, and different Caribbean countries and it is now in Madeira, northern Portugal and Spain (Ilharco *et al.*, 2005; Hermoso de Mendoza *et al.*, 2008).

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Disease syndromes caused by CTV fall into three types depending on virus strain and rootstock-cultivar combination (Moreno *et al.*, 2008). The so-called "severe" CTV strains are associated with (i) quick decline (QD) or tristeza of citrus species grafted onto sour orange or lemon [*C. limon* (L.) Burm. f.], (ii) the stem pitting (SP) syndrome characterized by the presence of elongated pits on branches of trees grown on tristeza-tolerant rootstocks, which reduces plant vigour and fruit quality, and (iii) seedling yellows (SY), that is observed by biological indexing. Mild CTV strains also occur in field trees but they cause barely detectable symptoms, usually only observed on indicator plants such as Mexican lime [*C. aurantifolia* (Christm.) Swing.] (Hancevic *et al.*, 2013).

In nature, CTV exists as a mixture of sequence variants, subisolates or recombinants (Broadbent *et al.*, 1996; Kong *et al.*, 2000; Rubio *et al.*, 2001; Matos *et al.*, 2013), displaying high levels of genetic and phenotypic diversity. Biological data appeared inconsistent when analyzing virus variability, while typing methods which target the coat protein (CP) gene are more reliable (Niblett *et al.*, 2000). Single-strand conformation polymorphism (SSCP) is further used to estimate the genetic diversity within and between isolates (Rubio *et al.*, 1996; Sambade *et al.*, 2002), and worldwide occurring isolates of CTV are now grouped in seven clusters based on the analysis of their CP gene (Nolasco *et al.*, 2009) associated with different symptoms (Hancevic *et al.*, 2013).

In Greece, the citrus industry represents an important branch of the economy, with an annual production of 1.3 million t; citrus crops are cultivated in 58 000 ha consisting mainly of oranges [*Citrus sinensis* (L.) Osbeck] (70%) and lemons (17%), grafted onto sour orange rootstock (Hellenic Statistic Authority, 2006). CTV is regulated as a quarantine pathogen in the country; all areas of citrus cultivation are surveyed, and eradication measures are applied when the virus is found (Dimou and Coutretsis, 2009). CTV was firstly detected in 2000, in imported sweet orange cv. Lane Late trees grafted on Carrizo citrange, in Argolida (North East Peloponnese) (Dimou *et al.*, 2002) and Chania (Crete) (Dimou and Coutretsis, 2009). Analyses of the nucleotide sequences of the p20 gene of these isolates showed high homology with the Spanish mild T385 isolate (Varveri, 2006; Shegani *et al.*, 2012). A similar isolate was collected in 2005, from sweet orange trees of the cv. Washington navel and Navelina in Arta prefecture (North West Greece) (Barbarossa *et al.*, 2007). In 2007, CTV was detected in an orchard in Scala Laconias (South Peloponnese), and subsequently in 2008, in Attiki and Chalkidiki, in greenhouses producing ornamental citrus Calamondin [*Citrofortunella microcarpa* (Bunge.) Wijnands]. In 2009, a more virulent strain was obtained from old lemon and mandarin trees from the "lemonwood" (an area with ca. 100-year-old lemon trees) in Poros (North East Peloponnese), and this strain clustered in group 3b (Nolasco *et al.*, 2009) according to its CP and p20 gene sequence analysis (Malandraki *et al.*, 2011). Although quarantine measures were applied, limited dissemination has occurred from the initial foci in Argolida and Chania (Dimou and Coutretsis, 2009).

Typing of CTV strains is considered a key element for predicting disease spread and adopting more efficient control strategies (Moreno *et al.*, 2008). In this study, SSCP analysis of the CP gene of CTV and specific sequencing of selected variants was used to characterize the population structure of CTV isolates occurring in the main disease foci in Greece.

Materials and methods

Virus isolates

Ten CTV isolates were initially used in the study in order to select distinct isolates for further molecular analysis. Most isolates were obtained from field plants; five from sweet orange trees in Chania, Crete (designated GR 1, 4-7) and two from lemon trees of the lemonwood in Poros (GR 8, 9). CTV isolate GR3 was obtained from a Mexican lime seedling grafted with a sweet orange isolate from Argolida that was previously characterized by Varveri (2006), and was maintained in an insect-proof greenhouse at the Benaki Phytopathological Institute (Athens, Greece). Field plants infected with these isolates were originally detected by the Greek quarantine system and sampled before eradication. Two isolates (CTV 10 and 11) from Calamondin plants originating from Italy were also included in the study. The presence of CTV in the infected plant material was confirmed in double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) tests with polyclonal antibodies (Agdia Biofords) according to the manufacture's procedures.

IC/RT-PCR, RT-PCR or PCR amplification of the coat protein gene

One step Reverse Transcription (RT)-Polymerase Chain Reaction (PCR) and Immunocapture (IC) RT-PCR were performed using the primers CTV1 (5′-ATGGACGACGAAACAAAGAA-3′) and CTV10 (5′-ATCAACGTGTGTTGAATTTCC-3′) amplifying a 672 bp product including the whole CP gene (Papayannis *et al.*, 2007).

Total RNA was extracted from fresh bark tissue (0.1 g) using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Subsequently, a single step RT-PCR was performed in 50 μL of a reaction mixture containing 2 mg of RNA, 10 mM Tris (pH8.8), 50 mM KCl, 0.08% Nonidet P-40, 2 mM $MgCl_2$, 200 nM each of the primers, 200 μM of each dNTP, 1U DreamTaq DNA polymerase (Thermo Scientific), 7.5U RNAguard (Amersham Pharmacia) and 7.5U MuLV reverse transcriptase (Perkin Elmer). RT was performed for 45 min at 38°C followed by PCR amplification with 2 min at 94°C, 30 cycles at 92°C for 30 s, 52°C for 30 s and 72°C for 45 s and finally 5 min at 72°C. PCR-products were separated in agarose gel electrophoresis (1%), stained with ethidium bromide and visualized under UV light. For IC/RT-PCR, RT-PCR tests were performed in tubes previously coated with anti-CTV IgG and ELISA extracts of the CTV infected samples without the addition of total RNA in the RT-PCR reaction mixture (Nolasco *et al.*, 2002). PCR tests were also applied to confirm the transformation of *Escherichia coli* colonies, using the same primers and conditions as described for the IC/RT-PCR and RT-PCR.

SSCP analysis of RT-PCR or PCR products

SSCP analysis was performed on the amplicons obtained by RT-PCR (of infected tissues) and on the PCR products (of recombinant *E. coli* colonies), in order to separate distinct virus variants. The amplified product (1 to 3 μL) was mixed with 9 μL of denaturing buffer [95% formamide, 20 mM EDTA, pH 8, 0.05% bromophenol blue) placed for 5 min at 90°C and then chilled on ice. Denatured products were electrophoresed in a non-denaturing 8% polyacrylamide gel (Bio-Rad Mini-Protean II, Bio Rad Laboratories) for 3 h at 200 V, 4ºC, using TBE (89 mM Tris-Borate, 2 mM EDTA, pH 8) as buffer (Rubio *et al.*, 1996). The gels were stained with GelStar (Lonza Bioscience) and visualized under UV light. PCR products displaying different SSCP patterns were considered different genomic variants (haplotypes) (Kong *et al.*, 2000).

Cloning and sequencing

Selected amplicons were TA-ligated into pTZ57R/T vector (InsTAclone PCR Cloning Kit, Thermo Fisher Scientific Inc.) and competent *E. coli* cells (Mach1™-T1^R, Invitrogen) were transformed according to manufacturer's instructions. Transformed colonies were selected by α-complementation on plates supplied with X-gal and ampicillin, according to standard procedures (Sambrook *et al.*, 1989). Transformation was confirmed by PCR amplification of the white colonies using primers CTV1/CTV10, and distinct clones were identified by SSCP analysis of at least 15 colonies per CTV isolate. Selected colonies were transferred to liquid medium and the plasmids harbouring selected variants were purified using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific Inc.) according to manufacturer's instructions.

Sequencing reactions were carried out at CCMAR, Universidade do Algarve, using the forward and reverse primers. Sequence alignment and clustering were performed using MEGA5 software package (Tamura *et al.*, 2011). Searches for recombination events among the sequences were carried out using the software package RDP (Martin *et al.*, 2010), which implements several algorithms for detecting recombination. The obtained sequences (Table 1) were analysed with the following reference sequences of the CP gene from the GenBank: 19–121, Portugal (AF184114); T36, Florida (M76485); VT, Israel (U56902); SY568, California (AF001623); and T30, Florida (AF260651). Sequences of B249, Venezuela and T3, Florida were provided by C.L. Niblett. These sequences are representative of each of the CTV phylogenetic groups proposed by Nolasco *et al.* (2009).

Results

IC/RT-PCR, RT-PCR and SSCP analysis of CTV isolates

IC/RT-PCR and RT-PCR of all samples resulted in amplicons of the expected size (672 bp) (results not shown). The preliminary assessment of these amplicons showed four different SSCP patterns (Figure 1a). No variability was observed among field isolates

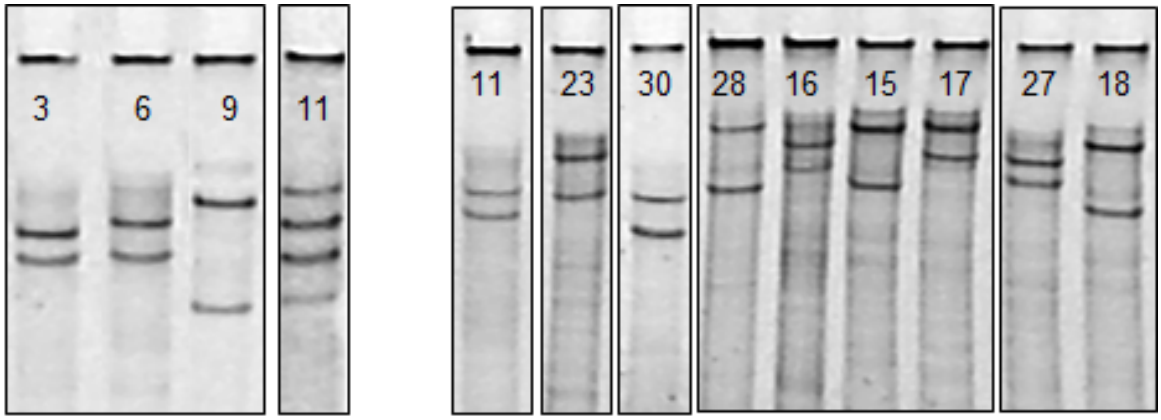

Figure 1. SSCP patterns obtained from the RT-PCR amplicons of (a) infected plants (GR3, GR6, GR9, CTV11) and (b) their haplotypes subsequently sequenced. *Citrus tristeza virus* (CTV) isolates: GR3, Mexican lime grafted with the isolate from Argolida (haplotype 11); GR6, Sweet orange from Chania (haplotypes 23, 30); GR9, Lemon from Poros (haplotypes 28, 16, 15, 17); and CTV11, Calamondin from Italy (haplotypes 27, 18).

originating from the one area and for each isolate in the patterns of the amplicons produced by either IC/RT-PCR or RT-PCR (results not shown).

The CP genes of the CTV isolates GR3, GR6, GR9 and CTV11, representing different SSCP patterns (Figure 1a), were subsequently cloned. A clear predominance of a single SSCP pattern consisting of two conspicuous bands was obvious in all isolates; for isolate GR3 this was the sole haplotype detected (GR3-11). CTV isolate GR6 consisted of two haplotypes; GR6-30 represented the one observed for the 87% of the analysed clones, while GR6-23 was observed in only 13% of them. Clones of isolate GR9 consisted of 60% of the haplotype GR9-15, 2% of the GR9-28, while one haplotype (6.5%) for each of the GR9-16 and GR9-17 was also present. Isolate CTV11 was difficult to clone; among 40 clones tested only four were successfully transformed showing two haplotypes CTV11-27 (75%) and CTV11-18 (25%) (Figure 1b).

Sequences and comparative analysis of the CTV coat protein

From each isolate one clone representing each haplotype obtained was chosen for sequencing. Nine sequences obtained were submitted to the GenBank with the accession numbers: KF196264, KF196265, KF196266, KF196267, KF196268, KF196269, KF196270, KF196271, KF196272 (Table 1). After excising the two terminal parts 20 bases long that corresponded to the primers, search for evidence of recombination was carried out among the new sequences, and the representative international isolates that were considered for comparisons. A strong recombination signal was obtained with several of the algorithms implemented in the RDP software for sequence GR9-16 in the stretch between positions 440 and 630. According to RDP, the backbone of GR9-16 derives from GR9-17 (group 3b) and the recombination stretch is close to the group 5 Greek sequences. Except for GR9-16, the remaining CTV sequences were aligned, their pairwise distances determined according to the Kimura 2 parameters model, and the resulting distance matrix was used to reconstruct a phylogenetic tree (Figure 2). GR9-16 was not included, as this evolutionary model is not suitable for calculating distances among sequences evolving through recombination.

All sequences matched with the expected sequence of the CTV coat protein. However, they were distributed clearly among three CP gene clusters, with high bootstrap values. The only SSCP variant detected in isolate GR3 (GR3-11) was grouped with the reference sequence from phylogenetic group M, while both variants of isolate GR6 (GR6-23, GR6-30) were clustered in group 5. The CP sequence variants of isolate CTV11 (CTV11-27, CTV11-18) were grouped into two different clusters (groups 5 and 3b), while those of isolate GR9 (GR9-15, GR9-17, GR9-28) were in group 3b.

Table 1. The GeneBank accession numbers and geographical locations in Greece and Italy of nine isolates and variants of *Citrus tristeza virus* (CTV).

CTV isolate	Host	Geographic origin	Haplo-type	Accession No.
GR3	Mexican lime	Argolida	11	KF196264
GR6	Orange	Chania	23	KF196269
			30	KF196272
GR9	Lemon	Poros	15	KF196265
			16	KF196266
			17	KF196267
			28	KF196271
CTV11	Calamondin	Italy	18	KF196268
			27	KF196270

Discussion

CTV epidemiology in a region is highly influenced by the susceptibility of the cultivated citrus varieties, the composition and the population dynamic of the aphid vector fauna, and the predominant virus isolates (Moreno *et al.*, 2008). In Greece, the prevalent use of sour orange rootstock provides very many CTV susceptible trees, while the aphid vectors *A. gossypii, A. spiraecola* and *T. aurantii* are widespread (Tsitsipis *et al.*, 2007). Therefore, the strain profile of CTV is a key factor for predicting future spread of the disease. In this study, SSCP analysis was employed to assess the population structure and sequence variability of the CP gene of three Greek and one Italian CTV isolates. Due to the quarantine status of CTV in Greece, a limited number of isolates were available; however the genetic diversity of the virus was still obvious.

Two of the field isolates analysed, Poros (GR9) and the Italian (CTV-11) isolate, had typical quasi-species structures consisting of sequence variants, with the one being predominant (Holland *et al.*,

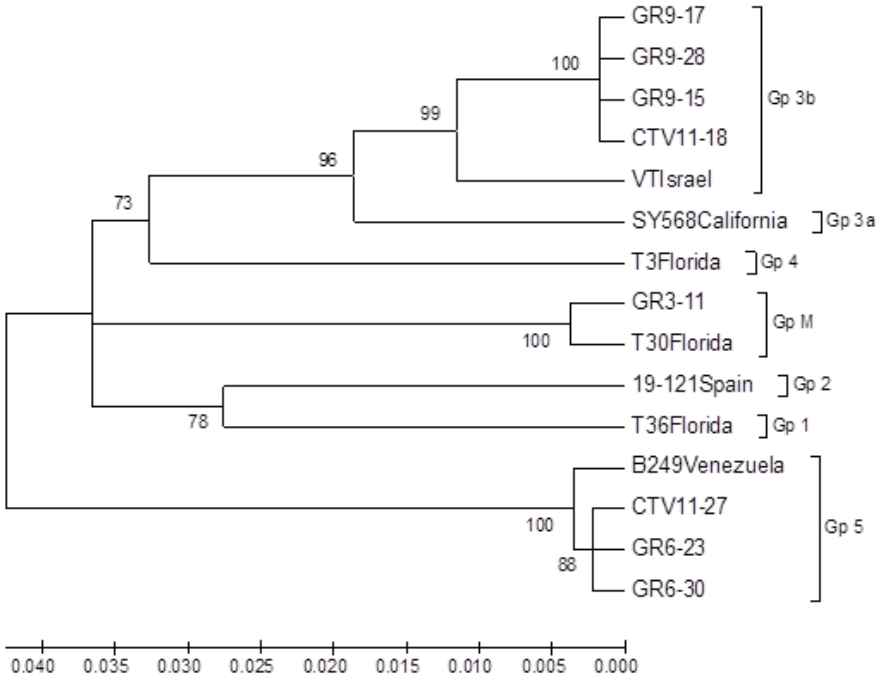

Figure 2. Neighbour-joining tree obtained from the matrix of pair-wise distances (nucleotide, Kimura 2-parameter) between the CP gene sequences of nine isolates of *Citrus tristeza virus* (CTV) from Greece and Italy. Bootstrap values obtained from 1000 replications are presented. Nodes with bootstrap values lower than 70% are not individualized. Group names as proposed by Nolasco *et al.* (2009) are indicated on the right. Nucleotide distance is represented in the horizontal bar.

1982). The prevalent haplotype in the Poros isolate was classified in group 3b, in agreement with previous studies (Malandraki *et al.*, 2011). However, we also detected, at a low concentration, a recombinant that implies the natural presence of variants of group 5, as well. The 100-year-old lemonwood may represent a distinct environment for CTV strain variability and spread. Infected lemon plants grafted on sour orange remain symptomless with low CTV titre and uneven distribution of the virus (Moreno and Garnsey, 2010). Long standing CTV presence within individual trees may therefore remain unnoticed, favouring reinfection and changes such as mutation or recombination (Rubio *et al.*, 2001; Martin *et al.*, 2009; Roy and Brlansky, 2009; Melzer *et al.*, 2010; Biswas *et al.*, 2012). On the other hand, the poor colonisation of lemon by *A. gossypii* (Barbagallo *et al.*, 2007) and its low susceptibility to aphid transmission of CTV (Hermoso de Mendoza *et al.*, 1988) may be the reason for the restricted virus spread within the lemonwood population (Malandraki *et al.*, 2011).

In Chania, CTV was introduced (Dimou and Coutretsis, 2009) with propagation material infected with a strain closely related to the Spanish T385 (Varveri, 2006; Shegani *et al.*, 2012), which clusters in group M (Nolasco *et al.*, 2009). In our analysis, however, a field isolate from the same area (GR6) consisted of only haplotypes of group 5, suggesting that this variant was either overlooked in previous studies or represents another virus introduction. Severe CTV strains can be masked by milder ones (Broadbent *et al.*, 1996; Brlansky *et al.*, 2003). In this area, natural spread of CTV was reported (Dimou and Coutretsis, 2009; Shegani *et al.*, 2012) suggesting virus adaptation to the local aphid vectors (Moreno *et al.*, 2008). Aphid mediated transmission may alter strain profile as CTV subisolates differ in their transmissibility (Broadbent *et al.*, 1996; Ayllón *et al.*, 1999; Brlansky *et al.*, 2003; Huang *et al.*, 2005; Nolasco *et al.*, 2008). The *A. gossypii* that occurs in the area has been associated with long-range dissemination of the virus (Gottwald *et al.*, 1999) that may maximise this bottleneck (Nolasco *et al.*, 2008) and may have contributed to the emergence of haplotypes of group 5 in preference to the mild strains previously reported.

The slow evolution of CTV has resulted in low strain diversity, despite the extensive global exchange of budwood (Silva *et al.*, 2012). Sequence variants from groups M and 3b are dominant in the Mediterranean Basin, reflecting the major CTV sources of Spain for group M, and Israel for 3b (Djelouah *et al.*, 2009). In agreement with this, the original isolate introduced with Spanish budwood in Argolida and Chania was previously included in the Spanish T386 isolate (Varveri, 2006; Dimou and Coutretsis, 2009; Shegani *et al.*, 2012) that clusters in group M with mild isolates (Nolasco *et al.*, 2009). In our studies of the isolate from Argolida (GR3) we detected only a variant belonging to group M. However, in the isolate from Chania we only detected variants of group 5, which are not reported by other recipients of Spanish propagation material (Lbida *et al.*, 2004). Additionally, the predominant variant of the Italian Calamondin isolate (CTV-11) was also clustered in group 5, although only isolates corresponding to groups 3b and M were previously identified in Italian plant material (Barbarossa and Savino, 2004; Davino *et al.*, 2005; Abou Kubaa *et al.*, 2012). In the Mediterranean basin, strains clustering in group 5 are present in the field in Croatia, Cyprus, Morocco, Portugal, Syria and Tunisia (Djelouah *et al.*, 2009; Abou Kubaa *et al.*, 2012). The presence of these variants in Greece may reflect a different CTV introduction from a location at which diverse CTV isolates occur.

Overall, our analysis of the three Greek and one Italian isolates pointed to the predominance of variants clustered in groups 3b and 5, which harbour severe SP and SY isolates, as do isolates VT from Israel and B249 from Venezuela (Nolasco *et al.*, 2009). The presence of these variants, even at low incidence, may explain the citrus decline symptoms observed in Greece, in areas where only mild strains were previously reported (Dimou and Coutretsis, 2009). For CTV, the structure of a complex virus population, and occasionally the major (Sambade *et al.*, 2002) or minor (Cerni *et al.*, 2008) components of the mixture, determine field symptoms. On the other hand, the presence of potentially aggressive strains may be critical for the development of future epidemics, as these strains show greater fitness and become predominant in mixed infected plants (Moreno *et al.*, 2008), while their spread seems to be favoured by *T. citricida* over the mild strains (Rocha-Rena *et al.*, 1995; Niblett *et al.*, 2000; Halbert *et al.*, 2004; Matos *et al.*, 2013).

In Greece, quarantine measures are constantly applied in order to limit CTV spread. However, the spread of *T. citricida* will challenge the ability of our agricultural systems to quickly shift to resistant rootstocks, and particularly the efficiency of our quar-

antine and certification programmes against the increasing threats posed by severe CTV strains.

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